TITLE

TRICARBALLYLATE AND HYDROXYCITRATE: SUBSTRATE AND INHIBITOR OF ATP: CITRATE OXALOACETATE LYASE

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Summary
Citrate cleavage enzyme shows atypical kinetics at low chloride concentrations, but normal kinetics at high chloride concentrations. Tricarballylate can be demonstrated both by the disappearance of CoA and, in the presence of hydroxylamine, by the appearance of a hydroxamate. This indicates that tricarballylyl – CoA is formed in the reaction. When hydroxamate formation is used to assay activity, the apparent Km for tricarballylate is about three times greater than that for citrate, while the maximum reaction velocity with tricarballylate is about 90% of that observed with citrate.

CONCLUSION OF STUDY

The experiments suggest that the apparent affinity of Citrate Cleavage Enzyme for (-)-Hydroxycitrate is more than 100 times greater than its apparent affinity for Citrate.

HOW THIS STUDY IS RELEVANT TO OUR PRODUCT

These experiments demonstrate some of the mechanisms of action of (-) HCA, furthers our understanding of how (-)-HCA works and leads to a better determination of its safety.

The knowledge of this inhibition of Citrate Cleavage Enzyme by (-)-HCA was used by subsequent researchers to determine the benefits and safety of (-)-HCA.

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Tricarballylate and Hydroxycitrate: Substrate and Inhibitor of ATP: Citrate Oxaloacetate Lyase[1]

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Citrate cleavage enzyme shows atypical kinetics at low chloride concentrations, but normal kinetics at high chloride concentrations. Tricarballylate is a substrate for citrate cleavage enzyme. The reaction with tricarballylate can be demonstrated both by the disappearance of CoA and, in the presence of hydroxylamine, by the appearance of a hydroxamate. This indicates that tricarballylyl-CoA is formed in the reaction. When hydroxamate formation is used to assay activity, the apparent K_m for tricarballylate is about three times greater than that for citrate, while the maximum reaction velocity with tricarballylate is about 90% of that observed with citrate. One of the stereoisomers of hydroxycitrate is a powerful inhibitor of citrate cleavage enzyme.

The cleavage of citrate is catalyzed by ATP: citrate oxaloacetate lyase (E.C. 4.1.3.8) (citrate cleavage enzyme) according to the stoichiometry (1, 2):

$$\text{citrate} + \text{CoA} + \text{ATP} \rightarrow \text{acetyl-CoA} + \text{oxaloacetate} + \text{ADP} + P_i \quad (1)$$

The enzyme from rat liver has been purified highly be Takeda and associates (3). These workers showed that the enzyme reacts with ATP to form a phosphoryl enzyme which can be isolated by gel filtration. Incubation of the phosphoryl enzyme with citrate results in the loss of its phosphoryl group. Incubation of the phosphoryl enzyme with citrate and CoA results in the formation of acetyl-CoA and oxaloacetate. When the enzyme is incubated with ATP, Mg^{++} ions, and citrate, in the absence of CoA, it forms citryl enzyme which can be isolated by gel filtration. The citryl enzyme loses its citryl group on incubation with CoA, and this process is accompanied by the formation of acetyl-CoA and oxaloacetate (4, 5). A phosphorylated enzyme intermediate was also demonstrated by Plowman and Cleland (6). Recently Walsh and Spector (7) showed that synthetically prepared citryl phosphate acts as a substrate for citrate cleavage enzyme. The rate of cleavage of the citrate moiety of citryl-phosphate was found to be similar to that of the overall reaction. These findings suggest that the following steps are involved in the complete reaction:

$$\text{enzyme} + \text{ATP} \xrightarrow{Mg^{++}} \text{phosphoryl-enzyme} + \text{ADP} \quad (2)$$

$$\text{phosphoryl-enzyme} + \text{citrate} \rightarrow \text{citryl-phosphate-enzyme} \quad (3)$$

$$\text{citryl-phosphate-enzyme} \rightarrow \text{citryl-enzyme} + \text{orthophosphate} \quad (4)$$

$$\text{citryl-enzyme} + \text{CoA} \rightarrow \text{acetyl-CoA} + \text{oxaloacetate} + \text{enzyme} \quad (5)$$

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Citryl CoA is a hypothetical intermediate of the reaction which can be fitted into the above sequence by dividing reaction (5) into two steps:

citryl-enzyme + CoA
→ citryl-CoA-enzyme (6)

citryl-CoA-enzyme
→ acetyl-CoA + oxaloacetate + enzyme (7)

Attempts to isolate citryl-CoA from enzyme reaction mixtures have failed so far. Eggerer and Remberger (8) showed that synthetically prepared citryl-CoA is cleaved by the enzyme, but at a rate which is much slower than the complete reaction. A similar finding was reported subsequently by Srere and Bhaduri (9).

The present paper shows that tricarballylate (propane 1,2,3-tricarboxylate) can replace citrate as a substrate for citrate cleavage enzyme. The reaction with tricarballylate can be demonstrated both by the disappearance of CoA and by the formation of tricarballylyl hydroxamate. In the absence of hydroxylamine the product of the reaction with tricarballylate is probably tricarballylyl CoA.

The stereoisomer of hydroxycitrate obtained from *Garcinia cambogia* (10) inhibits citrate cleavage enzyme when either citrate or tricarballylate is used as substrate. Tricarballylate acts as a weak inhibitor of citrate cleavage enzyme when citrate is used as substrate.

METHODS

Preparation of enzyme. Livers from rats which had been starved for 2 days and which were then fed a diet high in fructose for 3 days were used as starting material. It was shown previously that the level of citrate cleavage enzyme is very high in the liver of rats subjected to this regimen (11). The purification was carried out using the first two steps of the procedure of Inoue *et al.* (3). These involve ammonium sulfate precipitation between 0-30% saturation, and DEAE-column chromatography. The specific activity of citrate cleavage enzyme so obtained was 1-2 μmoles citrate cleaved per mg protein per min. Inoue *et al.* reported an activity of 0.3 μmoles per mg protein per min at this stage of purification. In view of the excellent reproducibility of the procedure of Inoue *et al.*, the difference in specific activity is probably due to the higher activity of our starting material.

Measurement of enzyme activity. The enzyme was first activated by incubation in one of the complete reaction mixtures described below, but which lacked ATP, at 23° for at least 15 min. If the activation prior to the assay was omitted, duplicate assays performed over a period of 2 hours showed relatively poor reproducibility. Excellent reproducibility was observed when the enzyme was activated prior to assay.

Assay 1. Citrate was used as substrate, and malate dehydrogenase and DPNH were used to reduce the oxaloacetate formed in the reaction (2). The reaction mixture contained 20 mM potassium citrate, 20 mM $MgCl_2$, 0.34 mM CoA, 93 mM Tris,HCl buffer, pH 8.2, 10 mM dithiothreitol, 3.33 mM ATP, 0.13-0.16 mM DPNH, malate dehydrogenase (0.33 unit/ml), and citrate cleavage enzyme (1-1.7 milliunits/ml). The final volume was 3.0 ml and the temperature was 23°. The assay was started by addition of ATP after the activation procedure described above, and the reaction was followed by measuring the decrease in absorbance at 340 mμ.

Assay 2. Citrate was used as substrate and hydroxylamine was used to trap the acetyl CoA formed in the reaction as hydroxamate (1). The reaction mixture contained 20 mM potassium citrate, 20 mM $MgCl_2$, 0.7 mM CoA, 175 mM Tris, HCl buffer, pH 8.0, 200 mM hydroxylamine, 10 mM dithiothreitol, 10 mM ATP, and citrate cleavage enzyme. The final volume was 1.0 ml and the temperature was 37°. The activation procedure described above was carried out at 23° in the absence of hydroxylamine and ATP. The hydroxylamine was then added and the reaction was started by adding the ATP. The hydroxylamine solution added was 2 M. It was prepared from 4 M hydroxylamine hydrochloride by adding an equal volume of 14.7% KOH just before use. When diluted tenfold the pH of this solution was 7.8-8.0. The reaction was stopped after 20 min and the hydroxamate color was developed as described by Inoue *et al.* (3). Hydroxamate formation was linear with time for at least 30 min. Under the conditions used, authentic acetohydroxamate showed a millimolar extinction coefficient of 0.99 at 520 mμ.

Assay 3. Tricarballylate was used as substrate and hydroxylamine was used to trap the acyl intermediate formed in the reaction as hydroxamate. The reaction mixture was identical to that used in Assay 2 except that citrate was replaced with tricarballylate. The ratio (extinction coefficient

for citryl hydroxamate)/(extinction coefficient for acetohydroxamate) is 0.82 at 546 mμ (12). In the absence of authentic tricarballylyl hydroxamate we assumed that the ratio (extinction coefficient for tricarballylyl hydroxamate)/(extinction coefficient for acetohydroxamate) is the same as above.

Sources of chemicals, enzymes, and rats. Hydroxycitrates isolated from *Garcinia cambogia* and from *Hibiscus sabdariffa* were generous gifts from Dr. Y. S. Lewis (10). We express our sincere thanks for his generosity. Our thanks also go to Dr. A. F. Tucci for his kind gifts of homocitric lactone, homoisocitric acid, and homoaconitic acid (13). ATP, DPNH, and CoA were obtained from Sigma Chemical Co., St. Louis, Missouri, and from P L Biochemicals Inc., Milwaukee, Wisconsin. Tricarballylate was obtained from Aldrich Chemical Co. Inc., Milwaukee, Wisconsin. Malate dehydrogenase was obtained from Boehringer Mannheim Corp., New York, New York. All other chemicals were of reagent grade. Rats of the Charles River CD strain were obtained from Charles River Breeding Laboratories, Inc., Wilmington, Massachusetts.

RESULTS

Effects of chloride and prior activation on reaction velocity. The effect of citrate concentration on activity is dependent on the level of chloride present in the reaction mixture (Fig. 1). At 85 meq Cl^- the Lineweaver-Burk plot shows a bimodal character. Assuming this to be the result of two independent active sites, two apparent K_m values for citrate, 0.16 mM and approximately 5 mM, can be obtained at low and high citrate concentrations respectively. At 500 meq Cl^- the Lineweaver-Burk plot yields a straight line and a K_m value for citrate of 0.14 mM, while V_{max} is reduced by about one-half. Unlike the deviations commonly observed in Lineweaver-Burk plots, the lines for low chloride concentrations attain a constant slope which is less than the initial slope (Fig. 2).

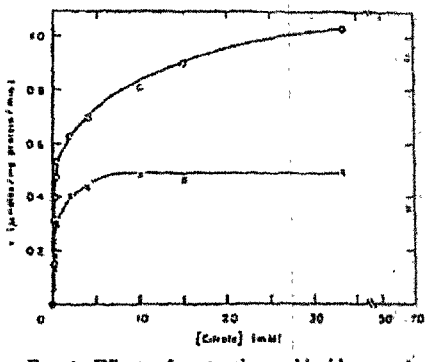

FIG. 1. Effect of potassium chloride on the citrate cleavage reaction. The conditions used were those of Assay 1, except that the citrate concentration was varied as shown. In addition the reaction mixture contained either 500 mM KCl (X), or the normal complement of chloride for this assay, namely 85 meq Cl^- (O).

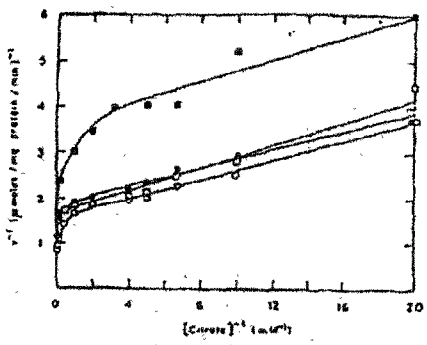

FIG. 2. Effect of dialysis and of prior incubation with citrate on activity of citrate cleavage enzyme. The stock solution of enzyme (specific activity about 1.0 unit/mg protein) was stored at 4° in 10 mM potassium citrate, 10 mM dithiothreitol, and 1 mM $MgCl_2$ at a pH of 7.8. The stock solution was divided into two portions, A and B. Portion A (hollow symbols) was diluted 20-fold just before use with a solution containing 10 mM potassium citrate, 10 mM dithiothreitol, and 150 mM Tris,HCl buffer, pH 7.8. Portion B (solid symbols) was dialyzed against 1000 vol of 1 mM $MgCl_2$, 1 mM dithiothreitol, and 10 mM Tris,HCl buffer, pH 7.8, at 4° for 18 hr. The dialyzed enzyme was diluted tenfold with the same medium and stored frozen until used. Portions A and B were then assayed before (□, ■) and after (○, ●) activation. The activation consisted of incubating the enzyme with the standard assay mixture less ATP at 23° for 60 min. Assay 1 was then started by adding the correct amount of ATP.

Citrate cleavage enzyme from rat liver loses activity on dialysis in the absence of citrate. This loss can be restored more or less fully by incubating the enzyme with the complete reaction mixture less ATP (Fig. 2). This observation is noted in passing; it is of

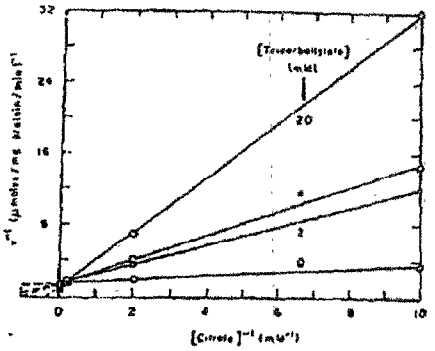

FIG. 3. Inhibition of citrate cleavage by tricarballylate. The conditions for Assay 1 were used, the concentration of citrate being varied as shown. In addition, the reaction mixture contained tricarballylate at the following concentrations: ○, none; △, 2 mM; □, 4 mM; and ◇, 20 mM.

importance when accurate measurements of total citrate cleavage activity are desired, as for example in a comparison of the activities of citrate cleavage enzyme and acetyl CoA carboxylase (14).

Effects of tricarballylate. When the citrate cleavage reaction is run in the presence of tricarballylate, the formation of oxaloacetate is inhibited in a competitive manner. The apparent K_i for tricarballylate is about 1 mM (range 0.5–1.5 mM) while the apparent K_m for citrate is about 0.13 mM (Fig. 3).

Tricarballylate also forms a product in the citrate cleavage reaction. This can be demonstrated as follows. The enzyme is first incubated for 30 min in the presence of the substrates used for Assay 1, but with tricarballylate in place of citrate. No oxidation of DPNH is observed under these conditions. Citrate is now added for the second phase of the incubation, and the oxidation of DPNH is followed at 340 mμ (Fig. 4). The reaction rate observed during the second phase (curve F) is much slower than that observed with controls in which ATP is omitted from the first incubation, but in which tricarballylate and citrate are present in various combinations and for various times (curves B, C, D, E). Moreover, the cleavage of citrate, as measured by Assay 1, is biphasic in the presence of tricarballylate. The slower phase sets in sooner when the enzyme is first incubated with tricarballylate, ATP, CoA, and $MgCl_2$ (curve F), than when tricarballylate or ATP are omitted from the first incubation. A simple interpretation of this finding is that the enzyme is catalyzing a reaction with tricarballylate, CoA, and ATP, which leads

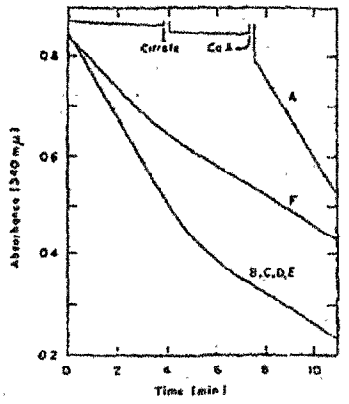

FIG. 4. Effect of tricarballylate on citrate cleavage reaction. The final reaction mixture contained 33.3 mM citrate, 20 mM $MgCl_2$, 90 mM Tris, HCl buffer, pH 8.3, 3.3 mM ATP, 0.043 mM CoA, 0.13 mM DPNH, citrate cleavage enzyme (specific activity about 1.0 unit/mg protein) and crystalline malate dehydrogenase (about 1.0 unit). The incubation temperature was 24°. To obtain tracing A, the enzyme was incubated with the above constituents, less citrate and CoA, for 15 min, and citrate and CoA were added at the times indicated. To obtain tracing B, the enzyme was incubated with the above constituents, less ATP, for 15 min, and ATP was added at zero time. To obtain tracing C, the enzyme was incubated with the same constituents as for B, plus 20 mM tricarballylate but less ATP, for 15 min, and ATP was added at zero time. To obtain tracing D, the enzyme was incubated with the above constituents, plus 20 mM tricarballylate but less ATP and citrate, for 15 min, and ATP and citrate were added at zero time. Tracing E was obtained in the same manner as curve D, except that the enzyme was incubated with tricarballylate for 30 min prior to adding ATP and citrate. Tracing F was obtained in the same manner as curve D except that the enzyme was incubated with 20 mM tricarballylate but less ATP and citrate, for 15 min; ATP was then added and the mixture was incubated for 30 min; 20 mM citrate was then added at zero time.

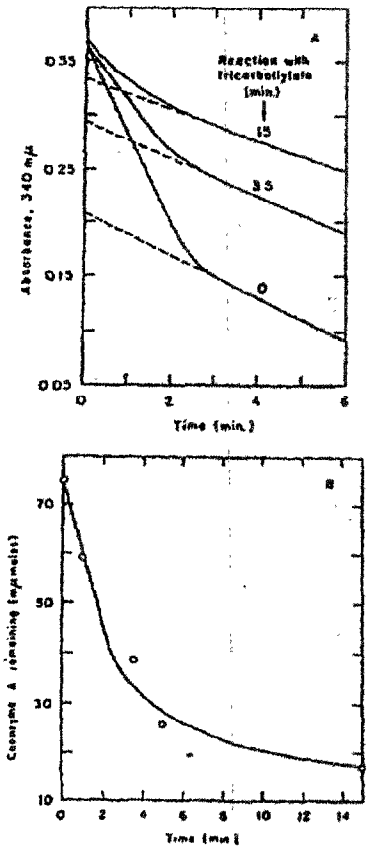

FIG. 5. Disappearance of free coenzyme A during reaction with tricarballylate. The incubation mixture contained 20 mM tricarballylate, 20 mM $MgCl_2$, 90 mM Tris.HCl buffer, pH 8.3, 0.025 mM CoA, 0.13 mM DPNH, citrate cleavage enzyme (specific activity 1.0 unit/mg protein) and crystalline malate dehydrogenase (about 1.0 unit). The final volume was 3.0 ml, and the incubation temperature was 24°. The above mixture was first incubated for 15 min, and the reaction was then started by adding 3.3 mM ATP. The reaction with tricarballylate was continued for the times indicated on the curves in *A*. Citrate (33.3 mM) was then added at zero time, and the citrate cleavage reaction was followed spectrophotometrically at 340 mμ as shown in *A*. To obtain *B* the limiting slopes of the curves in *A* were extrapolated to zero time, and the intercepts with the ordinate of a number of such extrapolations were plotted against the time for which the reaction with tricarballylate and ATP was run.

to the removal of CoA in the form of tricarballylyl CoA. According to this interpretation the longer the enzyme is incubated with tricarballylate, CoA, and ATP, the less oxaloacetate will be produced before the slow reaction sets in. This is indeed observed, as is demonstrated by the experiment shown in Fig. 5A. After the enzyme has been incubated with tricarballylate, CoA, and ATP for 15 min, relatively little oxaloacetate is formed from citrate before onset of the slow phase. More oxaloacetate is liberated after a similar treatment for 3.5 min, and most is liberated when citrate and tricarballylate are added together. The slow phase of oxaloacetate liberation (Fig. 5A) is probably a measure of the rate of breakdown of tricarballylyl CoA. If this is correct, extrapolation of the slow phase to zero time should provide a measure of the free CoA remaining after the incubation with tricarballylate. This extrapolation was carried out on the curves shown in Fig. 5A, as well as on similar curves obtained after 1 and 5 min of incubation with tricarballylate, with the results shown in Fig. 5B. Assuming our interpretation is correct, Fig. 5B shows that only 35% free CoA remains after 5 min of incubation with tricarballylate.

Enzyme (0.21 mg protein), 3.5 μmoles CoA, 3.5 μmoles ATP, 30 μmoles $MgCl_2$, 30 μmoles dithiothreitol, and 150 mM Tris-HCl buffer, pH 8.0, were mixed together in 0.9 ml and the absorbance of the solution was measured at 232 mμ using a light-path of 0.5 mm. Tricarballylate (0.1 ml of 200 mM) was added, and the change in absorbance at 232 mμ was followed using a light-path of 0.5 mm. The reaction stopped after about 35 min. About 1.5 μmoles thioester had formed, assuming a millimolar extinction coefficient at 232 mμ of 4.5. No thioester formation occurred when ATP or CoA or tricarballylate or enzyme were omitted from the reaction mixture.

When the citrate cleavage reaction is assayed by the hydroxamate method, it is found that a hydroxamate color is obtained when citrate is replaced by tricarballylate. Lineweaver-Burk plots show that the apparent K_m for tricarballylate is about 3 mM (this is referred to again later, see Fig. 9).

TABLE I

HYDROXAMATE FORMATION BY VARIOUS TRICARBOXYLIC ACIDS

Assay 3 was used, except that tricarboxylic acids were added as indicated. The reaction mixture contained 65 μg protein. A millimolar extinction coefficient of 0.82 at 520 mμ was used to calculate the amounts of hydroxamate formed

Substance added	Citrate		
	0	0.9 mM	14 mM
	Hydroxamate formed (μmoles/mg/min)		
None	0	200	430
Homo-citrate,[a] 38 mM	10	35	198
Homo-isocitrate, 32 mM	15	181	408
Homo-aconitate, 26 mM	16	192	380
(−)-Hydroxycitrate,[b] 3.5 mM	—	—	162
(+)-*Allo*-hydroxycitrate,[c] 5 mM	49	162	458
Tricarballylate			
2 mM	118	—	—
6 mM	200	—	—
30 mM	378	—	—

[a] Prepared from homo-citric lactone by saponification.
[b] From *Garcinia cambogia*.
[c] From *Hibiscus sabdariffa*.

TABLE II

INHIBITION OF CITRATE CLEAVAGE ENZYME BY HYDROXYCITRIC ACIDS

Assay 3 was used, except that tricarboxylic acids were added as indicated. The reaction mixture contained 60 μg protein.

Substance added	Citrate	
	0.3 mM	9 mM
	Hydroxamate formed (μmoles/min)	
None	243	635
(−)-Hydroxycitrate		
35 μM	85	440
(+)-*Allo*-hydroxycitrate		
50 μM	250	611
5000 μM	45	495

The apparent K_m for citrate in the hydroxamate assay is in the range of 0.5–1.0 mM.

Effects of various other tricarboxylic acids. Several analogs of citrate were tested for hydroxamate formation in the presence of citrate cleavage enzyme. Slight hydroxamate colors were obtained at high concentrations of homo-citrate, homo-isocitrate, homo-aconitate and *allo*-hydroxycitrate. These substances are also weak inhibitors of citrate cleavage (Table I). In contrast, a relatively powerful inhibition of citrate cleavage is observed in the presence of (−)-hydroxycitrate. The stereoselectivity of the inhibition of citrate cleavage by hydroxycitrates is demonstrated further in Table II, which shows that (−)-hydroxycitrate is a much more powerful inhibitor than (+)-*allo*-hydroxycitrate.

Inhibition by hydoxycitrate. In the presence of low concentrations of chloride the inhibition of citrate cleavage by (−)-hydroxycitrate is competitive at citrate concentrations below 1 mM (Fig. 6). At higher citrate concentrations the Lineweaver-Burk plots deviate from linearity. As has already been stated, this type of deviation is largely abolished in the presence of 0.3 M KCl (Fig. 7, see also Fig. 1). The K_m values for citrate are 70 μM and 200 μM at 85 and 385 meq Cl^- respectively. The corresponding K_i values for hydroxycitrate are 0.15 and 0.57 μM. It should be noted that K_m and K_i values in the presence of 85 meq Cl^- apply only to

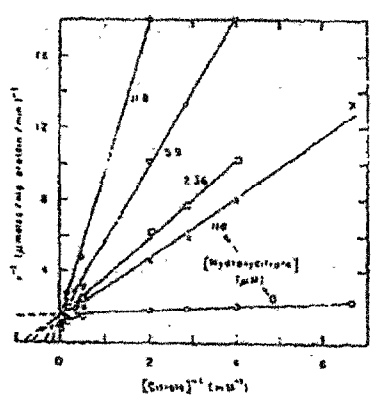

FIG. 6. Inhibition of citrate cleavage by (−)-hydroxycitrate. Assay 1 was used, the concentration of citrate being varied as shown. In addition the reaction mixture contained (−)-hydroxycitrate at the following concentrations: ○, none; ×, 1.18 μM; □, 2.36 μM; ▽, 5.9 μM; and ●, 11.8 μM.

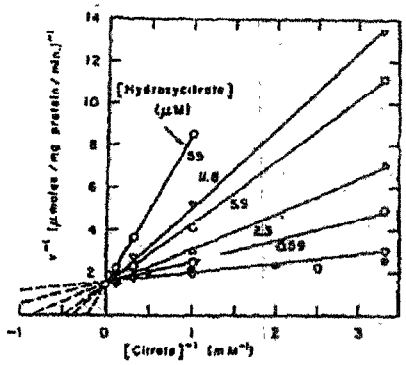

FIG. 7. Inhibition of citrate cleavage by (−)-hydroxycitrate. The conditions used were those of Assay 1, except that the citrate concentration was varied as shown. In addition the reaction mixture contained 0.3 M KCl, and (−)-hydroxycitrate at the following concentrations: ○, none; ◇, 0.59 μM; △, 2.3 μM; □, 5.9 μM; ▽, 11.8 μM, and ◯, 59 μM. The solid circles (●) show results obtained in the absence of (−)-hydroxycitrate, without added KCl but in the presence of the normal complement of chloride for this assay, namely 85 meq Cl⁻.

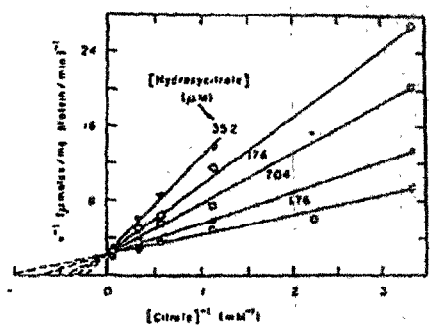

FIG. 8. Inhibition of citrate cleavage by (−)-hydroxycitrate. The conditions used were those of Assay 2, except that the concentration of potassium citrate was varied as shown. In addition the reaction mixture contained (−)-hydroxycitrate at the following concentrations: ○, none; △, 1.76 μM; □, 7.04 μM; ◇, 17.6 μM; and ▽, 35.2 μM.

citrate concentrations of less than about 1 mM. The above measurements were performed using the malate dehydrogenase assay.

Hydroxycitrate shows similar effects in the

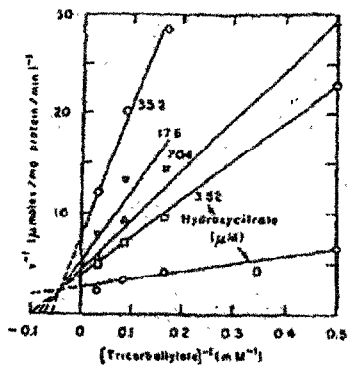

FIG. 9. Inhibition of tricarballylyl hydroxamate formation by (−)-hydroxycitrate. Assay 3 was used. In addition the reaction mixture contained (−)-hydroxycitrate at the following concentrations: ○, none; □, 3.52 μM; △, 7.04 μM; ▽, 17.6 μM; and ◇, 35.2 μM.

hydroxamate assay (Fig. 8). The assay involves a Cl^- concentration of 328 meq; it is therefore an assay in the presence of "high" chloride concentrations, and straight lines are obtained in the Lineweaver-Burk plot. The hydroxamate assay yields a K_m for citrate of 1.0 mM and a K_i for hydroxycitrate in the range of 4–9 μM. These experiments suggest that the apparent affinity of citrate cleavage enzyme for (-)-hydroxycitrate is more than 100 times greater than its apparent affinity for citrate.

Figure 9 shows that tricarballylyl hydroxamate formation is also inhibited by hydroxycitrate. The inhibition is of the mixed variety. The K_m value for tricarballylate is 3.0 mM. The K_i values for hydroxycitrate, computed by the method of Dixon (15), fall in the range of 2–18 μM over the range of hydroxycitrate concentrations shown in Fig. 9.

DISCUSSION

One possible explanation for the somewhat unusual type of kinetic behavior observed at low concentrations of chloride is that citrate cleavage enzyme possesses two types of active site, one with a high and the other with a low apparent affinity for citrate. It is necessary to postulate further that the site with

the low affinity for citrate is inhibited or masked at high chloride concentrations. A second possibility is that the enzyme possesses, say, two identical sites which show negative interaction. According to this model, citrate bound to one site lowers the affinity for citrate of the other site. Chloride increases the negative interaction, that is to say chloride lowers the affinity of the second site for citrate to a point where it no longer functions. A third possibility is that the enzyme occurs in two forms, one form possessing a higher affinity for citrate and a lower V_{max} than the other. An increase in the concentration of chloride shifts the equilibrium from the low affinity form to the high affinity form. Citrate may also influence the equilibrium between the two forms. Lastly, the possibility that the bimodal Lineweaver-Burk plots are due to the presence of isozymes with different K_m values cannot be eliminated in view of recent observations that isozymes of many enzymes are difficult to resolve. If correct, this possibility implies that one isozyme of citrate cleavage enzyme is inhibited by chloride while the other is not.

Similar types of deviation from Michaelis-Menten kinetics have been observed for a number of enzymes, including urease (16), fumarase (17), esterase (18–21), aldolase (22), and purine nucleoside phosphorylase (23). Possible models which may account for such deviation have been discussed in particular by Hearon *et al.* (24), Adler and Kistiakowski (20), and Kim *et al.* (23). The effect of chloride on citrate cleavage enzyme was first reported by Plowman and Cleland (6). These workers also discussed various models which may account for the kinetics observed in the presence and absence of chloride. They found that bromide and fluoride have the same effect as chloride, while acetate does not. Using conditions similar to those shown in Figs. 1 and 6, we have found that acetate also serves to normalize the kinetics of the enzyme.

The activation of tricarballylate by citrate cleavage enzyme can be demonstrated both by the disappearance of CoA and by the formation of tricarballylyl hydroxamate. Formation of the hydroxamate is dependent on the presence of CoA. For example, under the conditions described in Table I, no hydroxamate formation whatsoever can be observed from either citrate or tricarballylate when CoA is omitted. A direct demonstration of tricarballylyl CoA formation catalyzed by citrate cleavage enzyme will be reported subsequently. The reactions of tricarballylate support a reaction sequence for citrate cleavage enzyme in which citryl-CoA is an intermediate (Reaction 6). They do not support a sequence in which Reaction 5 is divided into the steps:

$$\text{citryl-enzyme} \longrightarrow \text{acetyl-enzyme} + \text{oxaloacetate} \quad (8)$$

$$\text{acetyl-enzyme} + \text{CoA} \longrightarrow \text{acetyl-CoA} + \text{enzyme} \quad (9)$$

The observation that tricarballylyl hydroxamate formation proceeds at a rate similar to acetohydroxamate formation (Table I), indicates that the postulated citryl-CoA formation cannot be dismissed as a slow side reaction. It does not definitely rule out reactions 8 and 9 as the major pathway, and reaction 6 (and also tricarballylyl CoA formation) as a side reaction. Nevertheless our experiments with tricarballylate strongly favor reactions 6 and 7 as being part of the normal, overall reaction sequence.

The discovery of the powerful inhibition of citrate cleavage enzyme by (−)-hydroxycitrate provides a valuable tool for the study of the metabolic role of the citrate cleavage reaction (11, 25). As will be reported later, hydroxycitrate and related compounds are excellent inhibitors of fatty acid synthesis.

Since this manuscript was submitted a report has appeared describing the enzymatic formation of tricarballylyl CoA (26). The experiments with citryl phosphate, which are mentioned in the introduction, have been described in greater detail (27).

The decrease in affinity for substrate with increasing substrate concentration, which is shown by some enzymes, has been termed "negative cooperativity" by Koshland. Glyceraldehyde-3-phosphate dehydrogenase (28) and cytidine triphosphate synthetase (29) fall into this category of enzyme.

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